U.S. PATENT APPLICATION

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Invention: A METHOD OF MODULATING MELANIN PRODUCTION

SPECIFICATION

A METHOD OF MODULATING MELANIN PRODUCTION

This application claims priority from Provisional Application No. 60/398,725, filed July 29, 2002, the content of which is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates, in general, to melanin production and, in particular, to a method of modulating melanin production and to compounds and compositions suitable for use in such a method.

BACKGROUND

Zinc alpha-2-glycoprotein (ZAG) is a plasma glycoprotein that was named for its electrophoretic mobility and for its ability to be precipitated by Zn salts (Burgi and Schmid 1961). It is a member of the immunoglobulin gene superfamily and has a three-dimensional structure that is highly homologous to MHC class I and II molecules (Sanchez *et al* 1999). ZAG has been detected immunohistochemically in normal secretory epithelial cells of breast, prostate, and liver, in salivary, bronchial, gastrointestinal, and sweat glands (Tada *et al* 1991; Hale *et al* 2001), and in normal stratified epithelia including epidermis (Lei *et al* 1997; Brysk *et al* 1997b). ZAG mRNA is expressed in a similar distribution (Freije *et al* 1991). Consistent with its production by secretory epithelium, ZAG is present in most body secretions and constitutes 2.5% and 30% of the proteins present in saliva and seminal fluid respectively (Poortsmans and Schmid 1968). Plasma or serum levels of ZAG have been described to vary with age, with reported values ranging from 0.9 - 3.5 mg/dl (fetal) to 7.8 - 12.1 mg/dl (young adults) to 18-30 mg/dl (normal men aged 51-70) (Jirka *et al* 1974; Jirka *et al* 1978; Hale *et al* 2001). ZAG accumulates in breast cyst fluids to 30-50-fold

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plasma concentration (Bundred *et al* 1987; Sanchez *et al* 1997) and is over-expressed in 40-50% of breast carcinomas (Bundred *et al* 1987; Sanchez *et al* 1992; Diez-Itza *et al* 1993). It has recently been shown that ZAG is produced in high amounts by most prostate carcinomas, resulting in elevated serum levels of ZAG in prostate cancer patients (Hale *et al* 2001). ZAG has also been identified in epidermal malignancies, including squamous and Merkel cell carcinomas, with lesser expression in basal cell carcinomas (Brysk *et al* 1997b; Lei *et al* 1997). Little is known about mechanisms regulating ZAG expression by tumors, however ZAG production by normal epithelial tissues was shown to be increased by treatment with androgens, corticosteroids, interferon-α, or TGF-α (Lopez-Boado *et al* 1994; Brysk *et al* 1997; Brysk *et al* 1997b).

The normal functions of ZAG are unclear, however ZAG has been isolated from the urine of human cancer patients with cachexia and can function as a lipid-mobilizing factor. ZAG purified from human or mouse serum or human cancer patient urine induces lipolysis resulting in glycerol release and also increases lipid utilization in both human and mouse adipocytes (Hirai *et al* 1998). ZAG activates a GTP-dependent adenylate cyclase activity on adipocyte membranes, increasing cellular cAMP levels (Hirai *et al* 1998). This may potentially lead to activation of multiple cellular pathways, however further details of its mechanism of action are unknown. Using a panel of murine tumors, Todorov *et al* (1998) quantitated ZAG production *in vitro* and showed that cachexia induction in mice bearing these tumors *in vivo* correlated with their ZAG production. Whether ZAG has additional biological activities in addition to cachexia induction is unknown.

To further investigate biologic properties of ZAG, stable transfectants of recombinant human (rh)ZAG were created in the B16F10 murine melanoma cell line. The effect of ZAG transfection on melanin production was determined *in vitro* and *in vivo*. The effect of exogenous ZAG on melanin production by parent B16 cells and melan-A primary melanocytes was determined. Finally, the effect

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of ZAG on tyrosinase expression and activity was determined. Taken together, these studies show that ZAG inhibits melanin production in both normal and malignant melanocytes. Mechanisms include post-transcriptional effects on tyrosinase protein, with the potential for additional indirect effects. These studies resulted in the identification of a previously unknown biologic function of ZAG and have made possible a method of modulating melanin production and thereby preventing and/or decreasing pigmentation of skin and hair due to increased melanin production.

SUMMARY OF THE INVENTION

The present invention relates generally to melanin production. More specifically, the invention relates to a method of modulating melanin production. The invention further relates to compounds and compositions suitable for use in such a method.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: B16-rhZAG clones demonstrate decreased melanin production *in vitro*. A. Visual inspection of media in stacked culture plates shows decreased melanin production by B16-rhZAG clones 10XA8 (lower plate) and 3G12 (middle plate) compared to B16-V cells (top plate) approximately 24 hours after achieving confluence. Eosin-stained cytocentrifuge preparations of B16-rhZAG clones 3G12 **(C)** and 10XA8 **(D)** also show decreased numbers of heavily melanized cells relative to B16-V cells **(B)** when examined during logarithmic growth.

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Figure 2. Decrease in melanin production by B16 cells *in vitro* correlates with ZAG over-expression. 5×10^5 cells were plated in 11 ml of phenol red-free media per 100 mm dish 96 hours prior to beginning absorbance measurements and reached confluence approximately 72 hours after plating. ZAG concentrations peaked at 47 hours post-confluence for 3G12 cells (**A**). Spectrophotometric analysis of culture supernatants (**B**) shows a significant lag in melanin production by B16-rhZAG clone 3G12, with peak A_{600} at 58 hours post-confluence. Decreased melanin production by B16-rhZAG clone 10XA8 was not detectable at the time points analyzed in these experiments. Media appeared black visually when A_{600} was ≥ 0.100.

Figure 3: Exogenous ZAG decreases melanin synthesis by B16-V cells *in vitro*. A. 10 - 100 µg/ml exogenous rhZAG inhibits the production and/or secretion of melanin into culture supernatant by B16-V cells. Results shown are representative of four experiments performed.

Figure 4: ZAG over-expression decreases melanin synthesis *in vivo* through post-transcriptional effects on tyrosinase protein. A ferrous ion uptake stain shows abundant melanin production (blue color) in B16-V cells (**A**), with near total absence of reactivity in murine tumors derived from B16-rhZAG clone 3G12 (**B**). Incubation of frozen tumor sections with L-DOPA shows strong tyrosinase activity (black color) in B16-V tumors (**C**) compared to almost total lack of tyrosinase activity in B16-rhZAG tumors (**D**). Immunoperoxidase staining with αPEP7 antiserum specific for tyrosinase showed decreased reactivity (red color) with B16-rhZAG tumors (**F**) relative to B16-V tumors (**E**). No staining is seen in B16-V (**G**) or B16-rhZAG (**H**) tumors using normal rabbit serum as a negative control.

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Figure 5: Exogenous ZAG inhibits melanin production by melan-A primary melanocytes *in vitro*. The indicated amount of ZAG was added to 6 well plates seeded with 0.165×10^6 melanA cells and cultured for 96 hours prior to harvesting the cells for assessment of melanin production by absorbance at 600 nm, histologic examination, and Western blot. **A.** ZAG inhibited melanin production in a dose-dependent fashion as determined by absorbance measurements of cell lysates. Data shown is the mean ± range of 2 independent experiments. Three additional experiments that used differing numbers of cells and different ZAG concentrations also showed a dose-dependent inhibition of melanin production. **B.** A Western blot detected with αPEP7 anti-tyrosinase antiserum demonstrates that tyrosinase protein is decreased in melan-A cells treated with 150 mg/ml ZAG as compared with PBS-treated control cells. **C, D.** Eosin-stained cytocentrifuge preparations show decreased numbers of heavily melanized cells in ZAG-treated (150 μg/ml) (panel D) vs. control melan-A cultures (panel C).

DETAILED DESCRIPTION OF THE INVENTION

Problems with increased or aberrant skin pigmentation can be psychologically devastating when widespread. Such changes may still cause significant distress even when localized to small area of the skin, especially when aberrant pigmentation involves the face and/or hands. The color of skin and hair depends on the amount of eumelanin that it contains. Eumelanin is produced by melanocytes through a series of chemical reactions that are regulated by the enzyme tyrosinase.

The present invention results from studies demonstrating that ZAG can inhibit melanin production by melanocytes. Increased levels of ZAG supplied as purified protein or through transfection and endogenous expression decreases the

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levels of tyrosinase protein and appear to decrease the activity of any residual tyrosinase enzyme. Application of ZAG to normal melanocytes *in vitro* results in marked decreases in melanin synthesis by these cells.

The present invention thus relates to a method of inhibiting melanin synthesis. The present method is useful in clinical situations of hyperpigmentation due, for example, to sun-exposure, inflammation, or scarring or to disorders with congenital or acquired proliferations of melanocytes. ZAG is produced by epidermal keratinocytes, thus it can be applied locally to the epidermis as well as systemically.

Decreases in skin and hair pigmentation due to aging begin with decreased melanin synthesis by existing melanocytes. ZAG concentrations in human blood increase substantially with age (Hale et al, Clin. Can. Res. 7:846 (2001)). Interfering with the action of ZAG locally on melanocytes can be used in a method of preventing age-related loss of hair and skin pigmentation. Furthermore, inhibition of naturally produced ZAG can allow restoration of pigmentation in skin and hair to decrease susceptibility to UV-induced cancers and to reverse age-related changes in hair and skin color.

ZAG decreases melanin synthesis by decreasing the amount and activity of tyrosinase in both normal and malignant melanocytes. Tyrosinase is a tumor-associated antigen in melanoma that has been investigated as a target for immunotherapy. Modulation of tyrosinase protein levels by ZAG can be useful in enhancing the efficacy of immunotherapy targeting tyrosinase.

ZAG can be formulated as a composition using any of a variety of physiologically acceptable carriers, excipients or diluants. The compostion can be, for example, in a cream, gel or lotion for topical use or can be present as a liquid, e.g., a sterile liquid when used systemically. The composition can also be present in dosage unit form, e.g., as a tablet or capsule.

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The data presented in the Example that follows show that ZAG regulates melanin production in B16 melanoma cells, a widely used model of melanocyte function. The generalizability of the anti-melanogenic functions of ZAG is further demonstrated by its inhibitory effect on melanogenesis by melan-A normal

5   murine melanocytes *in vitro*. ZAG is produced by normal epidermal keratinocytes, where its expression increases with cellular differentiation (Lei *et al* 1997). Others have previously demonstrated that keratinocyte-derived factors influence melanocyte behavior, including melanocyte proliferation, dendricity, and total melanin production (Gordon *et al* 1989). Thus taken together, the data

10   provided herein indicate that epidermal-derived ZAG plays a role in normal regulation of melanin production *in vivo*, in addition to its previously described role in cancer cachexia.

The studies described in the Example that follows demonstrate that B16 tumor cells that are either transfected to strongly express ZAG or treated with

15   exogenous ZAG have decreased melanin production *in vitro*. B16 tumors strongly expressing ZAG also have decreased growth and form amelanotic tumors *in vivo*. Despite qualitatively similar levels of tyrosinase mRNA expression, ZAG-transfected B16 (B16-rhZAG) tumors had decreased levels of tyrosinase protein and minimal tyrosinase activity. Purified ZAG also decreased melanin production

20   by B16-V cells *in vitro* and decreased tyrosinase activity in B16-V tumor sections *in vitro*. Taken together, these results demonstrate that ZAG inhibits melanin synthesis in B16 cells at a post-transcriptional level. However, ZAG appears to decrease tumor cell growth and melanin synthesis *in vivo* more strongly relative to its effects *in vitro*, indicating that ZAG may also act through other indirect

25   mechanisms *in vivo*. ZAG similarly decreases melanin production in primary murine melanocytes *in vitro*.

Moderate to high levels of ZAG are required to achieve significant inhibition of melanin synthesis. B16 cells are normally highly melanized despite

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their production of low levels of murine ZAG (Todorov *et al* 1998). The numbers of heavily melanized cells are decreased similarly in ZAG-transfected 10XA8 and 3G12 cultures (Figures 1C,D). However the accumulation of melanin in the media decreases much more in 3G12 cultures that produce approximately twice the ZAG produced by 10XA8 cultures (Figures 1A, 2A, 2B). Indeed, although visual decreases in secretion of melanin into the media by 10AX8 can be observed at some time points compared with vector-transfected cells (Figure 1A), secretion of melanin as measured by A_{600} of media at other timepoints is not significantly decreased in 10XA8 (Figure 2B). These observations may indicate that a threshold amount of ZAG may be required for simultaneously decreasing both melanogenesis and melanin secretion. However, the observation that ZAG-transfected tumor cells that make melanin *in vitro* are amelanotic *in vivo* (Figure 4B) demonstrates that the concentration of ZAG required to inhibit melanin synthesis is readily achieved at tumor sites *in vivo*. A variety of tumor types derived from secretory and stratified epithelium, including squamous cell carcinomas, have been shown to produce measurable amounts of ZAG (Hale *et al* 2001; Lei *et al* 1997; Lei *et al* 1999; Bundred *et al* 1987; Sanchez *et al* 1992; Diez-Itza *et al* 1993). It has previously been shown that tumor -produced ZAG can contribute to elevated systemic (serum) ZAG levels in prostate cancer patients (Hale *et al* 2001).

Higher levels of exogenous rhZAG are required to achieve similar levels of tyrosinase inhibition when compared to ZAG production by rhZAG-transfected cells. This may reflect very high local concentrations adjacent to rhZAG-producing cells or may indicate that ZAG also affects tyrosinase activity through indirect mechanisms that require metabolism or changes in gene expression, protein synthesis or half-life of ZAG or other proteins. The *in vivo* studies that show essentially complete inhibition of melanogenesis in tumors of ZAG-transfected B16 cells *in vivo* compared with only partial inhibition *in vitro* also

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suggest that ZAG may inhibit melanin synthesis by indirect mechanisms. This may occur through effects on other cells that are present *in vivo* in addition to direct effects that ZAG has on the tumor cells themselves *in vitro*. Alternatively, *in vivo* growth conditions may have generated and selected for new tumor cell

5   clones with resistance to ZAG and reduced production of melanin by alternative mechanisms.

The data presented show that ZAG expression also inhibits the growth of B16-rhZAG tumors *in vivo*. ZAG has previously been shown to inhibit the proliferation of the Tu-138 oral squamous cell carcinoma cell line, when the cells

10   are plated directly onto immobilized ZAG (Lei *et al* 1999). This implies that ZAG has additional biologic effects on tumor cells beyond stimulating lipolysis and melanin synthesis.

α-melanocyte stimulating hormone (α-MSH) has previously been shown to regulate the expression of tyrosinase mRNA, in addition to having other

15   neuroimmunomodulatory effects (Lipton and Catania 1997; Ottaviani *et al* 1997). While α-MSH was not measured directly, the studies presented in the Example provide no evidence for ZAG-induced changes in tyrosinase gene transcription. They do show a clear and direct effect of ZAG on level and activity of tyrosinase protein. However, decreased melanin synthesis by melanoma tumor cells could

20   potentially arise from inhibition in multiple pathways. ZAG may influence melanin production by B16 melanoma cells by additional mechanisms in addition to effects on tyrosinase protein.

Since TNF-α has previously been shown to inhibit melanin synthesis by human primary melanoma and B16 melanoma cells (Swope *et al* 1991; Martinez-

25   Esparza *et al* 1998; Martinez-Esparza *et al* 1999), it was important to determine whether ZAG acted via a mechanism involving TNF-α. TNF-α was undetectable in the supernatant of both B16-V and B16-rhZAG cells cultured *in vitro* and TNF-

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α mRNA could not be detected in either B16-V or B16-rhZAG cells by RT-PCR (data not shown). However, small amounts of TNF-α "spiked" into the supernatant from B16-V and B16-rhZAG cells could be detected quantitatively, indicating that these supernatants do not contain substances that interfere with detection of TNF-α protein. These findings indicate that ZAG inhibits melanin synthesis by B16 cells via a TNF-α-independent mechanism. ZAG may, however, be a TNF-mimetic or ZAG- and TNF-α-dependent pathways may share downsteam effector pathways.

Certain aspects of the invention can be described in greater detail in the non-limiting Example that follows.

EXAMPLE

Materials and Methods

<u>Construction of ZAG-Producing Murine Cell Lines</u>

A full-length human ZAG cDNA including the endogenous secretory signal sequence was cloned from human liver using RT-PCR and primers corresponding to bp 3-21 and bp 938-920 (GenBank D90427). The construct sequence was verified by automated DNA sequencing. Full length human ZAG cDNA was inserted into the pCDNA3.1(-) Myc-His eukaryotic expression vector (Invitrogen, Carlsbad, CA) using restriction enzyme digestion and adapter ligation to ensure in-frame insertion relative to the myc and 6-His 3' epitope tags. Epitope-tagged recombinant human ZAG constructs were transfected into B16F10 (B16) murine melanoma cells (American Type Culture Collection, Rockville, MD). Stable transfectants were obtained by G418 selection then cloned by limited dilution. Parent and transfected cell lines were grown in DMEM + 10% fetal bovine serum at 37°C in an atmosphere supplemented with 5% CO_2. Culture supernatants were analyzed in triplicate for expression of ZAG by antigen capture ELISA using anti-ZAG mAb 1B5 (Sanchez *et al* 1997) as capture antibody. Bound

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rhZAG was detected using biotinylated anti-ZAG mAb 1H4 (Sanchez *et al* 1997),

streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch Labs, West

Grove, PA), and 3,3',5,5'-tetramethyl-benzidine substrate (Kirkegaard and Perry

Laboratories, Gaithersburg, MD). Standard curves were constructed using HPLC

5   purified rhZAG, quantitated by A_{280} (Burgi and Schmid 1961). The sensitivity of the

assay was 10 pg/ml. ELISA results were confirmed by SDS-PAGE and Western blot

detection of epitope-tagged recombinant human (rh) ZAG with the predicted molecular

weight of 46 kDa using anti-ZAG mAb 1B5 and/or the India His -Probe™ nickel-

horseradish peroxidase reagent (Pierce, Rockford, IL) specific for the 6-His epitope tag.

10   Vector-transfected cell populations (B16-V) were also derived and used as controls.

B16-V cells showed no evidence of reactivity with the anti-human ZAG mAbs used in

either antigen capture enzyme immunoassays or Western blots (Figure 1), although B16

cells have been shown to express small amounts of murine ZAG (Todorov *et al* 1998).

15   Analysis of Melanin Production

Melanin content of B16 cell lines was determined by microscopic

evaluation of eosin-stained cytocentrifuge preparations. To be considered heavily

melanized, cells had to contain >10 melanosome specks visible at a magnification

of x100 or ≥ 1 macro-melanosome. The majority of cells considered heavily

20   melanized had at least a "salt and pepper" appearance with many melanosome

specks present. Melanin production by B16 cell lines was also determined by

absorbance measurements (600 nm) of phenol red-free culture supernatants.

Melan-A primary murine melanocytes (from Dr. Vincent Hearing,

National Institutes of Health) were propagated in RPMI1640, 10% fetal bovine

25   serum, 2 mM glutamine, 100,000 U/L penicillin, 100 mg/L streptomycin sulfate,

200 nM tetradecanoyl phorbol acetate and seeded into 6-well plates for

measurements of melanin production. After 96 hours of culture with various

concentrations of ZAG, media was aspirated and cells were washed once in PBS,

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then solubilized in 200 μl 1M NaOH, and pipetted repeatedly to homogenize. Melanin absorbance was determined at 600 nm. Melanin content of melan-A cells was also determined by microscopic evaluation of eosin-stained cytocentrifuge preparations.

Animal and Tissue Studies

2×10^5 rhZAG or vector-transfected B16 tumor cells were implanted subcutaneously in the flank in groups of 5 syngeneic female C57BL/6 mice. Mice were weighed then sacrificed and serum was obtained at 21 days post-implantation, just prior to tumor-related death. Tumor size was measured using calipers. The concentration of tumor-produced human ZAG in the serum was measured by antigen capture ELISA as described above. Each serum sample was analyzed in quadruplicate for at least 2 independent dilutions and results were averaged. Samples of tumors for histologic analysis were snap-frozen in Tissue-Tek OCT compound (Sakura, Torrance, CA) and stored at -80°C.

Immunoperoxidase staining for tyrosinase protein was performed on frozen sections of B16 tumors using αPEP7 rabbit polyclonal antiserum (gift of Dr. Vincent J. Hearing) (Jimenez 1991; Aroca 1993). Other reagents included biotinylated goat anti-rabbit secondary antibody, avidin-biotin-horseradish peroxidase complexes (VectaStainABC; Vector Laboratories, Burlingame, CA) and Nova Red substrate (Vector Laboratories). Negative controls to ensure the specificity of staining included use of normal rabbit serum on B16 tumors and use of αPEP7 on 4T1 murine breast carcinoma tumors that do not produce melanin.

Ferrous ion uptake and L-DOPA histochemical stains were performed on frozen sections of B16 tumors as described (Sheehan and Hrapchak 1987). The ferrous-ion uptake stain is specific for pre-formed melanin, generating a blue color in proportion to the amount of melanin present. The L-DOPA stain provides an assay for tyrosinase activity. *In vivo*, tyrosinase catalyzes the oxidation of

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tyrosine to DOPA (3,4-dihydroxyphenylalanine) as well as the oxidation of DOPA to melanin through intermediate steps. When fresh frozen tissue sections are incubated with a buffered solution of DOPA, a melanin-like pigment is deposited in proportion to the degree of tyrosinase activity present (Sheehan and

5   Hrapchak 1987). A Nuclear Fast Red counterstain was used for both ferrous ion uptake and L-DOPA histochemical stains.

For Western blot determination of tyrosinase protein levels, protein extracts were prepared from five 20 μm frozen sections of B16-V or B16-rhZAG tumors or from cultured melan-A cells using 10 mM Tris pH 8.0, 150 mM NaCl,

10   1% Triton X-100, with protease inhibitors (Complete Mini EDTA-free Protease Inhibitor tablets, Roche Molecular Biochemicals, Indianapolis, IN). The protein content of tumor extracts was determined using the BCA^{TM} assay (Pierce). Equal amounts of protein by weight for tumor extracts or corresponding to equivalent numbers of cells for melan-A cells were analyzed by SDS-PAGE, transferred to

15   nitrocellulose, and reacted with αPEP7 anti-tyrosinase rabbit polyclonal antiserum or control rabbit serum followed by goat anti-rabbit immunoglobulin coupled to horseradish peroxidase. Bound antibody was detected by chemiluminescence (SuperSignal West Pico Chemiluminescence Substrate, Pierce).

RNA was isolated from tumor tissue using an RNeasy^{TM} kit (Qiagen,

20   Valencia, CA). Two μg of RNA was reversed transcribed using SuperScript (Gibco BRL, Rockville, MD) and the resulting cDNA was subjected to PCR for 15, 20, 25, and 30 cycles using Platinum Taq (Gibco BRL). Primers were selected to cross intron-exon boundaries and did not amplify genomic DNA. Primer sequences for tyrosinase (415 bp) were 5' CAG ATC TCT GAT GGC CAT 3' and

25   5' GGA TGA CAT AGA CTG AGC 3' (bp 101 - 118 and bp 515 - 498, GenBank NM_011661) and for GAPDH (214 bp) were 5' TCG TCC CGT AGA CAA AAT G 3' and 5' TGA CAA GCT TCC CAT TCT C 3' (bp 31 - 49 and bp 244 - 227, GenBank M32599). Products were analyzed on ethidium-bromide stained gels

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and ZAG to GAPDH product ratio was determined at 15, 20, 25, and 30 cycles for each sample.

Purification of ZAG

5   rhZAG was purified from supernatant of rhZAG-transfected 293 human kidney epithelial cells using a Ni-NTA affinity column (Qiagen) specific for the His epitope tag or by concentration from serum free media (293 SFM II^{TM}, Gibco BRL) using a 30 kDa MW cutoff filter. SDS-PAGE gels followed by Western blotting confirmed that the protein in these ZAG preparations was highly

10   immunoreactive with anti-ZAG mAb.

TNF-α Assays

TNF-α protein was detected by antigen capture enzyme immunoassay using the Duo-Set TNF-α kit (R&D Systems, Minneapolis, MN) according to the

15   manufacturer's protocol. The sensitivity of this assay is 15 pg/ml. TNF-α mRNA was detected by RT-PCR assays using primers corresponding to bp 301 - 318 and bp 527 - 510 of the GenBank NM_013693 sequence (5' TGT CTA CTG AAC TTC GGG 3' and 5' TCT TTG AGA TCC ATG CCG 3', respectively). The 560 murine astrocytoma cell line transfected with TNF-α (Sampson *et al* 1997) was

20   used as a control to insure appropriate detection of both TNF-α mRNA and protein under the conditions used.

Results

ZAG over-production decreases melanin production by B16 cells *in vitro*

25   *In vitro*, B16 melanoma cells typically produce melanin such that the culture supernatant turns visually black soon after cultures become confluent. Melanin production by B16-rhZAG clones 3G12 and 10XA8 that expressed ZAG

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at high levels was compared with that by B16-V cells. A clear and consistent decrease in the rate of melanin accumulation could be seen within cultures of B16-rhZAG vs. B16-V cells by visual inspection of media soon after cultures reached confluence (Figure 1A). These visual differences could no longer be

5   discerned at later timepoints when melanin accumulation was maximal (not shown). Eosin-stained cytocentrifuge preparations of B16-rhZAG clones 3G12 and 10XA8 and B16-V cells obtained from logarithmically growing cultures were also examined for the presence of the cytoplasmic dark specks that are indicative of melanin production. The amount of melanin present within the cytoplasm of

10   ZAG-transfected cells was significantly decreased when compared with vector-transfected cells (Figure 1B-D). Fewer B16-rhZAG cells contained visible melanin specks and less total melanin was present in the cytoplasm of B16-rhZAG cells that contained melanin specks. In particular, percentage of heavily melanized cells was significantly reduced in both B16-rhZAG clones analyzed

15   relative to B16-V cells ($16\pm1\%$ and $14\pm2\%$, mean $\pm$ SD respectively for 3G12 and 10XA8 vs. $45\pm2\%$ for B16-V; $p < 0.0003$). Thus high level expression of ZAG decreases melanin synthesis by individual B16-rhZAG cells.

ZAG over-expression decreases the rate of melanin accumulation *in vitro*

20   To further investigate the effects of ZAG over-expression on melanin accumulation during culture, serial measurements of both melanin and rhZAG present in the media from B16-rhZAG clones 3G12 and 10XA8 and B16-V cells were performed as a function of time. As shown in Figure 2A, the concentration of ZAG peaks at 47 hours post-confluence and decreases thereafter. Although 3G12 cells

25   produce melanin *in vitro*, cultures do not turn visibly black until 51 hours post-confluence, compared with 42 hours for 10XA8 and B16-V cells. Melanin accumulation in the media of the 3G12 cell line remains consistently below that of B16-V cells for all time points analyzed (Figure 2B). Decreases in melanin production

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by B16-rhZAG clone 10XA8 compared with B16-V cells were not detectable at the time points analyzed in these experiments. Whether the increasing black color of the media from 24 - 58 hours post-confluence results from increased melanin synthesis and/or from increased secretion of melanin into the culture medium is unknown. Eosin-stained cytocentrifuge preparations of 3G12, 10XA8, and B16-V cells made 68 hours post-confluence show relatively few melanin granules compared with those made from logarithmically growing cells, suggesting that melanin secretion may increase post-confluence. However, these cells are viable and proliferate and synthesize melanin when passaged. The A_{600} of the melanin-containing media decreases by the 68 hour point (Figure 2B), most likely as a result of further oxidation or breakdown of previously produced melanin. This decreased absorbance suggests that oxidized melanin and/or melanin breakdown products contribute less to the measured A_{600} as compared to newly synthesized or secreted melanin. The observation that the A_{600} of melanin-containing media from B16-V cultures decreases during cell-free culture at 37°C support this interpretation.

Exogenous ZAG decreases melanin synthesis by B16-V cells *in vitro*

The above studies indicate that ZAG over-production is sufficient to decrease melanin synthesis by B16-rhZAG melanoma cells that are genetically engineered to over-express ZAG. To determine whether similar effects could be obtained when ZAG is supplied exogenously, B16-V cells were treated with purified rhZAG at concentrations ranging from 10 - 100 µg/ml. As shown in Figure 3, addition of exogenous rhZAG also inhibited melanin production by B16-V cells *in vitro* in a dose-dependent fashion.

ZAG expression turns off melanin synthesis by B16 tumor cells *in vivo*.

In addition to producing melanin in culture, B16 cells also typically produce melanin *in vivo* and B16 tumors thus appear black when grown in mice.

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The growth and melanin production by B16-rhZAG clone 3G12 and B16-V cells *in vivo* were next compared in groups of 5 syngeneic mice. B16-rhZAG tumors showed significantly decreased growth at 21 days compared with B16-V tumors (mean tumor volume ± SEM: 289 ± 74 mm^3 vs. 943 ± 62 mm^3, p<0.0002).

5 rhZAG could be detected in the serum of mice with B16-rhZAG but not B16-V tumors (156 ± 70 ng/ml (mean ± SD) vs. undetectable). Increased ZAG production by B16-rhZAG tumors was sufficient to cause a mean weight loss of 15% in the group bearing rhZAG-transfected tumors (ending weights: B16-vector 20.6 ± 1.1 g; B16-ZAG 17.5 ± 0.8 g; p = 0.001) which remained significant when

10 corrected for differences in tumor volumes. However, although B16-rhZAG clone 3G12 can produce (albeit decreased amounts of) melanin *in vitro,* tumors derived from these cells show no evidence of melanin production *in vivo* and thus appear white grossly. Histochemical staining using the more sensitive ferrous ion uptake stain similarly shows almost total lack of melanin production in B16-rhZAG

15 tumors, as indicated by lack of blue reaction product (Figure 4A,B). This marked decrease in melanin production by B16-rhZAG cells *in vivo* contrasts with the comparatively modest decrease in melanin production seen in these cells *in vitro*.

ZAG affects tyrosinase activity at a post-transcriptional level

20   Melanin synthesis is controlled in part by regulating the levels and/or activity of the tyrosinase enzyme that is required for the final stages of melanin synthesis. To further investigate mechanism by which tumor-produced ZAG affects melanin synthesis, the presence of mRNAs encoding tyrosinase in B16-rhZAG and B16-V cells grown *in vitro* and tumors grown *in vivo* were analyzed

25 by RT-PCR. The amount of tyrosinase product relative to GAPDH product was similar in melanin-producing B16-V tumors vs. amelanotic B16-rhZAG tumors grown *in vivo* as well as in B16-rhZAG vs. B16-V cells grown *in vitro*. This suggests that the decrease in melanin production by ZAG-transfected B16 cells

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most likely occurs by a mechanism other than by transcriptional inhibition of tyrosinase mRNA production.

To determine how ZAG affects tyrosinase enzyme activity, frozen sections of B16-rhZAG and B16-V tumors were incubated with the tyrosinase substrate L-DOPA. Although strong tyrosinase activity can be observed in B16-V tumors (black color, Figure 4C), very little tyrosinase activity is present in B16-rhZAG tumors (Figure 4D). B16-rhZAG tumors show clear evidence of reactivity with αPEP7 polyclonal anti-tyrosinase antibody in immunoperoxidase assays (red color, Figure 4F), but their reactivity is decreased relative to B16-V tumors (Figure 4E). Neither B16-V nor B16-rhZAG tumors react with normal rabbit serum alone (Figure 4G,H), and αPEP7 is non-reactive with 4T1 breast carcinoma tumors that do not produce melanin, further confirming the specificity of the αPEP7 reagent. To further study the relation between tyrosinase protein levels and tyrosinase enzyme activity, Western blots were also performed using extracts from three different B16-V and B16-rhZAG tumors. Specific bands corresponding to tyrosinase protein were present in all six tumors studied. However the amount of tyrosinase was qualitatively decreased in at least 2 of the 3 B16-rhZAG tumors tested. No reactivity was seen in duplicate blots reacted with control rabbit serum or in lanes containing extracts of 4T1-rhZAG breast cancer tumors that do not express tyrosinase. Thus, ZAG over-expression appears to decrease but does not abrogate the expression of tyrosinase protein in B16 tumors, when examined both immunohistologically and by Western blot.

The almost total lack of tyrosinase activity in B16-rhZAG tumors (Figure 4D) despite the presence of immunohistologically detectable tyrosinase protein (Figure 4F) suggested that ZAG may also affect tyrosinase activity by other mechanisms, in addition to affecting levels of tyrosinase protein. To determine whether ZAG could directly affect the activity of tyrosinase protein, frozen sections of B16-V tumors were incubated with varying concentrations of

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exogenous rhZAG prior to addition of the tyrosinase substrate L-DOPA. Sections
incubated with 1 - 8 mg/ml rhZAG had a 25 − 50% decrease in color development
with L-DOPA. ZAG concentrations less than 1 mg/ml gave no visible inhibition
of the L-DOPA reaction. Taken together, the immunohistochemical and L-DOPA

5   studies clearly demonstrate that ZAG decreases melanin synthesis in B16
melanoma cells by post-transcriptional effects on the level and activity of
tyrosinase protein.

ZAG inhibits melanin synthesis by normal melanocytes

10   To determine whether ZAG also affects melanin synthesis by normal
melanocytes, the melanin content of melan-A primary murine melanocytes treated
with varying concentrations of ZAG was determined. As shown in Figure 5A,
ZAG decreased melanin synthesis by melan-A cells in a dose-dependent fashion,
with a mean inhibition of 68% at 150 µg/ml exogenous ZAG. The number of

15   heavily melanized cells seen in eosin-stained cytocentrifuge preparations was
decreased by ZAG treatment (Figure 5C, D). Finally, Western blots using αPEP7
anti-tyrosinase Ab (Figure 5B) demonstrated that 150 µg/ml ZAG markedly
decreased levels of tyrosinase protein in melan-A cells, similar to its effect in B16
cells. Thus these studies indicate that ZAG has similar effects on melanin

20   production in both normal and malignant melanocytes.

References

Aroca P, Urabe K, Kobayashi T, Tsukamoto K, Hearing VJ. Melanin biosynthesis
patterns following hormonal stimulation. J Biol Chem 268:25650-25655, 1993

25

Brysk MM, Lei G, Selvanayagam P, Brysk H, Tyring SK, Arany I. Modulation by
interferon-γ of zinc-α2-glycoprotein gene expression in human epithelial cell
lines. Anticancer Res. 17:3387-3392, 1997

19

Brysk MM, Lei G, Rajaraman S, Selvanayagam P, Rassekh CH, Brysk H, Tyring SK, Arany I. Gene expression of zinc-a2-glycoprotein in normal human epidermal and buccal epithelia. In Vivo 11:271-274, 1997b

5

Bundred NJ, Miller WR, Walker RA. An immunohistochemical study of the tissue distribution of the breast cyst fluid protein, zinc alpha2-glycoprotein. Histopathol. 11:603-610, 1987.

10   Burgi W, Schmid K. Preparation and properties of Zn α2-glycoprotein of normal human plasma. J Biol Chem. 236:1066-1074, 1961

Diez-Itza I, Sanchez LM, Allende MT, Vizoso F, Ruibal A, Lopez-Otin C. Zn-α2-glycoprotein levels in breast cancer cytosols and correlation with clinical,

15   histological, and biochemical parameters. Eur J Cancer 29A:1256-1260, 1993

Freije JP, Fueyo A, Uria J, Lopez-Otin C. Human Zn-α2-glycoprotein cDNA cloning and expression analysis in benign and malignant breast tissues. FEBS Lett. 290:247-249, 1991

20   Gordon PR, Mansur CP, Gilchrest BA. Regulation of melanocyte growth, dendricity, and melanization by keratinocyte-derived factors. J. Invest. Dermatol. 92:565-572, 1989

Hale LP, Price DT, Sanchez LM, Demark-Wahnefried W, Madden JF. Zinc

25   alpha-2-glycoprotein is expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer. Clin. Cancer Res7:846-853, 2001

20

Hirai K, Hussey HJ, Barber MD, Price SA, Tisdale MJ. Biological evaluation of a lipid-mobilizing factor isolated from the urine of cancer patients. Cancer Res. 58:2359-2365, 1998

Jimenez M, Tsukamoto K, Hearing VJ. Tyrosinases from two different loci are expressed by normal and by transformed melanocytes. J Biol Chem 266:1147-1156, 1991

Jirka M, Blanicky P, Cerna M. The Zn-alpha 2-glycoprotein level in human serum during ontogenesis. Clin. Chim. Acta 56:31-36, 1974

Jirka M, Blanicky P, Srajer J, Zwinger A, Jirasel JE. Human serum Zn-α2-glycoprotein in amniotic fluid. Clin Chim Acta 85:107-110, 1978

Lei G, Arany I, Selvanayagam P, Rajaraman S, Ram S, Brysk H, Tyring SK, Brysk MM. Detection and cloning of epidermal zinc-α2-glycoprotein cDNA and expression in normal human skin and tumors. J. Cell. Biochem. 67:216-222, 1997

Lei G, Brysk H, Arany I, Srinivasan G, Brysk MM. Characterization of zinc-α2-glycoprotein as a cell adhesion molecule that inhibits the proliferation of an oral tumor cell line. J. Cell. Biochem. 75:160-169, 1999

Lipton JM, Catania A. Anti-inflammatory actions of the neuroimmunomodulator α-MSH. Immunol. Today 18:140-145, 1997

Lopez-Boado Y, Diez-Itza I, Tolivia J, Lopez-Otin C. Glucocorticoids and

760515

androgens up-regulate the Zn-α2-glycoprotein messenger RNA in human breast cancer cells. Breast Cancer Res Treat. 29:247-258, 1994

Martinez-Esparza M, Jimenez-Cervantes C, Solano F, Lozano JA, Garcia-Borron JC. Mechanisms of melanogenesis inhibition by tumor necrosis factor-α in B16/F10 mouse melanoma cells. Eur J Biochem. 255:139-146, 1998

Martinez-Esparza M, Solano F, Garcia-Borron JC. Independent regulation of tyrosinase by the hypopigmenting cytokines TGFβ1 and TNFα and the melanogenic hormone α-MSH in B16 mouse melanocytes. Cell Molec Biol. 45:991-1000, 1999

Ottaviani E, Franchini A, Franceschi C. Pro-opiomelanocortin-derived peptides, cytokines, and nitric oxide in immune responses and stress: an evolutionary approach. Int Rev Cytol. 170:79-141, 1997

Poortmans JR, Schmid K. The levels of Zn α2-glycoprotein in normal human body fluids and kidney extracts. J. Lab. Clin Med. 71:807-811, 1968

Sanchez LM, Vizoso F, Diez-Itza I, Lopez-Otin C. Identification of the major protein components in breast secretions from women with benign and malignant breast diseases. Cancer Res. 32:95-100, 1992

Sanchez LM, Lopez-Otin C, Bjorkman PJ. Biochemical characterization and crystalization of human Zn-α2-glycoprotein, a soluble class I major histocompatibility complex ligand. Proc Natl Acad Sci USA 94:4626-4630, 1997

22

Sanchez LM, Chirino AJ, Bjorkman PJ. Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules. Science 283:1914-1919, 1999

Sampson JH, Ashley DM, Archer GE, Fichs HE, Dranoff G, Hale LP, Bigner DD.

5   Characterization of a spontaneous murine astrocytoma and abrogation of its tumorigenicity by cytokine secretion. Neurosurgery 41:1365-1372, 1997

Sheehan DC, Hrapchak BB. Theory and practice of histotechnology, 2nd Edition. Batelle Press, Columbus, OH. 1987, pp. 222-224

10

Swope VB, Abdel-Malek, Kassem LM, Nordlund JJ. Interleukins 1α and 6 and tumor necrosis factor-α are paracrine inhibitors of human melanocyte proliferation and melanogenesis. J Invest Dermatol. 96:180-185, 1991

15   Tada T, Ohkubo I, Niwa M, Sasaki M, Tateyama H, Eimoto T. Immunohistochemical localization of Zn α2-glycoprotein in normal human tissues. J Histochem Cytochem. 39:1221-1226, 1991

Todorov PT, McDevitt TM, Meyer DJ, Ueyama H, Ohkubo I, Tisdale MJ.
20   Purification and characterization of a tumor lipid-mobilizing factor. Cancer Res. 58:2353-2358, 1998

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All documents cited above are hereby incorporated in their entirety by reference.

25

23